NITROGEN-FIXATION IN LEGUMINOUS PLANTS. VII.

THE NITROGEN-FIXING ACTIVITY OF ROOT NODULE TISSUE IN MEDICAGO AND TRIFOLIUM.

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(One Text-figure.)

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Introduction.

Although the problem of symbiotic nitrogen fixation by leguminous plants and root nodule bacteria has been studied in great detail for nearly sixty years, we still know comparatively little about the quantitative side of this process, expressed as the amount of nitrogen fixed in unit time per unit of root nodule substance, and hardly anything about the influence of environmental factors on the degree of nodule-efficiency thus expressed. One reason for this is the fact that comparatively few investigators have made separate determinations of total dry matter and nitrogen in the root nodules and the rest of the plants separately, while most have contented themselves with analysing the roots and nodules together, or the tops only.

Wozak (1929) concluded from field experiments with seven species of legumes that nitrogen was fixed at a rate of 36 mgm. (in *Vicia faba*) to 98 mgm. (in *Pisum sativum*) per gm. dry nodule substance per day. He pointed out that these values are only approximate (actually somewhat too high) because they include an unknown amount of combined nitrogen taken from the soil.

Bond (1936) estimated that nodules of soy beans grown in sand fixed 24–28 mgm. nitrogen per gm. dry matter per day in young plants, and 7–8 mgm. or less in older plants. Similar figures for nodules of soy beans in sand culture were calculated by Wilson (1940) on the basis of experiments described elsewhere (Wilson and Umbreit, 1937). Earlier experiments by Whiting (1915), who recorded only the nitrogen contents and not the dry weights, suggest a similar activity of nodules in soy beans and cowpeas. Nodules of soy beans grown in water culture (Bond, 1941, Table 1) show a lower activity; a calculation according to Bond's formula (1936) indicates a fixation of only 4 mgm. nitrogen per gm. dry nodule substance per day from 29th July to 7th September, 1940. The figures given by Giöbel (1926) in his Tables 36 and 37 permit a similar calculation of the activity of nodules of soy beans grown in sand with varying doses of combined nitrogen. If we assume that the nodules, of which only the fresh weights are given, contain 20% dry matter, we find during the period from 8 to 12 weeks a daily fixation of 7·2–10·9 mgm. nitrogen per gm. dry nodule matter, falling to 5·8 mgm. with the highest dose of nitrate. Unfortunately the experiment did not include plants grown entirely with free nitrogen.

Chen and Thornton (1940) estimated that the active bacterial tissue in nodules of red clover fixed 3·90–4·59 mgm. nitrogen per cubic centimetre per day. It may be assumed (Bond, 1941) that the bacterial tissue accounts for roughly one-half of the whole nodule, and that this contains 20% dry matter of unity specific gravity; the fixation would then correspond to 9·8–11·5 mgm. nitrogen per gm. dry matter per day, a value only about one-fifth of what Wozak (1929) observed in the same species (55 mgm.). In soy bean nodule tissue Chen and Thornton found an even lower value.

There are some other experimental records from which we may calculate the overall fixation of nitrogen during the whole growth period per gm. dry nodule matter at the

end of the experiment. Jones and Tisdale (1921) found that the largest mass of nodule substance in soy beans, both absolutely and in proportion to the weight of the plant, developed at 21–24°C., but the highest actual yields of nitrogen were found at 27–30°C. Their data show also that the gain of nitrogen per gm. dry nodule increases with the temperature, from 814 mgm. at 21°C. to 2271 mgm. at 36°C. These figures, like Wozak's (1929), are only approximate, because they include some combined nitrogen taken from the soil.

From the data of Virtanen (1928) on peas and three species of clover grown in sand we may calculate the following gains of nitrogen in mgm. per gm. dry nodule matter:

Pisum sativum	994-2423 mgm.
Trifolium pratense	1484-2073 „
„ *repens*	1222-1702 „
„ *hybridum*	2324-4364 „

These figures show no correlation with the reaction of the sand medium (pH 5·0, 5·5 and 6·0). On the other hand, a series of experiments by the present author (Jensen, 1943) with lucerne and subterranean clover in sand and soil of varying reaction showed that the proportional weight of the nodules was generally higher, but the gain of nitrogen per gm. dry nodule substance lower, at pH 4·9–6·2 than at pH 6·7–7·5. The same observation was made in a later experiment with subterranean clover (Jensen, 1944).

Beside the fact that the species of plants and the range of reaction were not the same in Virtanen's and my own experiments, and that no allowance was made for the uptake of combined nitrogen from the medium, it was later observed (Jensen, 1946) that a partial molybdenum deficiency tends to lower the efficiency but to increase the proportional weight of the root nodules in lucerne. Experiments by Stephens and Oertel (1943), Lewis (1943), Oertel et al. (1946), and Anderson and Oertel (1946), have shown consistently that the availability of molybdenum depends on the reaction of the soil, and that its uptake generally increases with increasing pH. It is thus possible that my earlier results do not express only the direct effect of pH on the activity of the nodules, but also an indirect effect through its influence on the availability of the small amounts of molybdenum present.

New experiments have therefore been performed under conditions that should give a better understanding of the influence of reaction on the nitrogen-fixing efficiency of the root nodules. The possibility of partial molybdenum deficiency at the lower pH-ranges was ruled out by giving an adequate supply of this element, corrections were made for the combined nitrogen taken from the medium, and in most experiments the plants were harvested at several successive stages of growth. Comparative tests with plants given combined nitrogen were included in some cases.

Methods.

Lucerne (*Medicago sativa*, var. "Giant Upright") and subterranean clover (*Trifolium subterraneum*, var. "Mount Barker"), beside barrel medic (*M. tribuloides*) and white clover (*T. repens*) in a preliminary test, were grown in glazed earthenware pots of 6 in. diameter, holding 3 kgm. sand. Unless otherwise stated, a river sand of medium coarseness and pH 5·8–5·9 was used, with an acid nutrient solution; neutral or faintly alkaline reaction was produced by adding 0·1 or 0·2% calcium carbonate to the sand. Twelve to fifteen seeds, inoculated with effective strains of the appropriate root nodule bacteria, were sown, and after germination the seedlings were thinned to (usually) six or eight per pot. The surface of the sand was covered with a mulch of clean, coarse gravel in order to reduce evaporation and growth of algae. The pots were kept in a greenhouse and arranged in randomized blocks. The moisture content of the sand was kept as near as possible to 12% of the weight of the sand, normally by watering with distilled water; during periods of maximum evaporation it was sometimes necessary to use the local tap water which contains very little lime and did not seem to affect the reaction of the sand appreciably.

At harvesting, watering was omitted for a few days previously, the plant tops were cut off at the surface, the gravel mulch was removed, and the roots were separated from the damp sand by gentle shaking and screening through a wide-meshed sieve from which the loose root-fragments were collected as completely as possible. Some loss of small rootlets was unavoidable, but it is unlikely to represent any significant proportion of the total plant-substance. The roots from each pot were placed in a dish with water and cautiously separated from each other, and the nodules were counted on each individual root system, picked off, dried, and weighed. Nodules detached during the separation of the roots were divided between the plants in that pot. The roots were kept in a refrigerator at 3–5°C. between harvest and removal of the nodules. In this way the plant material from each pot was divided into the three fractions of tops, roots, and root nodules, which were dried at 96–98°C., ground finely, and analysed for total nitrogen by the Kjeldahl method. Materials from replicate pots, of which there were usually three, were combined for analysis.

The theoretically best estimate of the amount of combined nitrogen derived by the plants from the sand would have been furnished by a series of control pots with nodule-free plants harvested at the same time as each set of inoculated plants, but this was not technically possible. As a second choice the amounts of nitrate and ammonia formed in unplanted pots, with and without calcium carbonate, were determined at intervals corresponding to the harvests. The method of Richardson (1938) was used, on somewhat larger samples of sand, usually 200 gms. The quantities of $(NO_3 + NH_4)$–N found after 8 to 18 weeks varied in the commonly used sand between 0·5 and 4·5 p.p.m., or 1·5–13·5 mgm. per pot. This plus the nitrogen content of the seed was subtracted from the gross nitrogen content of the plants to give the net gain of nitrogen. The "mobilizable" nitrogen appeared to be a fairly reliable measure of the amount of combined nitrogen becoming available, since similar quantities of nitrogen (6·2–13·3, average 10·8 mgm.) were found in wheat plants grown for 80 days in sand without addition of nitrogenous nutrients.

Experimental.

Preliminary Experiments.—Four species of legumes were grown in acid and alkaline sand and harvested only once, as in earlier experiments (Jensen, 1943). Lucerne and subterranean clover, ten plants per pot, were sown on 9th August, 1943, and harvested after 86 and 106 days, respectively. The nutrient solution contained 0·25 gm. KH_2PO_4; 0·25 gm. $CaCl_2$; 0·1 gm. $MgSO_4$; 0·05 gm. $FeCl_3$; and 2·5 mgm. $MnSO_4$, $ZnSO_4$, $CuSO_4$, Na_2MoO_4, and $Na_2B_4O_7$, all per pot; the last five constituents are in the following collectively referred to as "minor salts". The alkaline sand was given 0·2% $CaCO_3$. In this series alone the pots contained only 2·5 kgm. sand, and there were only duplicate pots of subterranean clover. Barrel medic and white clover were sown on 27th August, 1943, and harvested after 80 and 101 days. The results are seen in Table 1. In this and subsequent tables the significance of differences between yields at acid and alkaline reaction is expressed by the *t*-test (Fisher, 1946). Where only two yields are compared, the degree of significance is shown by the following symbols:

\+ : P = 0·05–0·02
\+ + : P = 0·02–0·01
\+ + + : P < 0·01

When more than two means are compared, the differences necessary for significance at the five, two and one per cent. points (P = 0·05, 0·02 and 0·01) are stated.

The figures show that only in *Medicago tribuloides* is the gain of nitrogen, actual as well as per gm. dry nodule, significantly lower at acid reaction. In the other plants the variation between replicate pots was considerable, and the gains of nitrogen are not significantly different, except that the gain per gm. dry nodule in subterranean clover is slightly higher at alkaline reaction. As found before (Jensen, 1943), the average numbers of nodules on lucerne and barrel medic are much lower in acid than in alkaline sand, but the weight of the whole nodule-fraction is less strongly affected, owing to a larger average size of the nodules in acid sand. Text-fig. 1 shows two selections of typical

TABLE 1.

Nitrogen Fixation by Four Species of Leguminous Plants in Acid and Alkaline Sand.

		Lucerne.		Subterranean Clover.		Barrel Medic.		White Clover.	
pH of sand, initial		5·7	7·2	5·7	7·2	5·0	7·4	5·0	7·4
„ „ „ final		4·7–4·8	7·1–7·3	4·3–4·4	7·2–7·3	4·8–5·2	7·5–7·6	4·3–4·5	7·6–7·8
Dry matter, gm. per pot, mean.	Tops ..	3·52	3·73	6·06	7·62	1·68	7·07	4·96	5·98
	Roots ..	3·12	3·53	1·23	1·71	0·37	1·37	0·88	1·03
	Nodules ..	0·130	0·145	0·309	0·344	0·095	0·132	0·105	0·125
Percentage of N in dry matter.	Tops ..	3·03	3·06	2·22	2·40	2·23	2·45	2·25	2·78
	Roots ..	2·07	2·04	2·35	2·34	1·69	2·24	2·27	2·06
	Nodules ..	6·14	8·33	6·43	7·34	3·61	5·40	5·82	5·30
Total N in plants, mgm.		178·9	198·2	183·1	248·1	47·1	211·0	137·4	194·0
Net gain of N, mgm.		176·2	193·7	175·9	239·1	38·7	204·1	131·0	189·1
Do. per gm. dry nodule-substance		1360	1352	569	698	407	1598	1248	1513
Nodules per plant.	Mean ..	11·7	62·3	65·7	49·6	10·5	36·2	64·8	77·3
	S.E.* ..	±0·90	±4·24	±4·86	±2·96	±1·68	±2·76	±6·96	±7·30
Significance of differences between:									
Net gains of N		–		–		+++		–	
Do. per gm. dry nodule-substance in acid and alkaline sand ..		–		+		+++		–	

* S.E. = standard error, expressed as the standard deviation divided by the square root of the number of plants.

nodules from lucerne. In the two clovers the reaction has little effect on either the numbers or the aggregate weight of the nodules; subterranean clover even shows a slightly but significantly higher mean number in acid sand.

The acidity of the acid sand increased strongly during the growth period. The clovers even acidified the sand to pH 4·3–4·4 without the gains of nitrogen being significantly lowered.

Another preliminary experiment was designed to show at what pH-level the effect on nodule numbers and nitrogen fixation becomes significant. A fine yellow hill sand

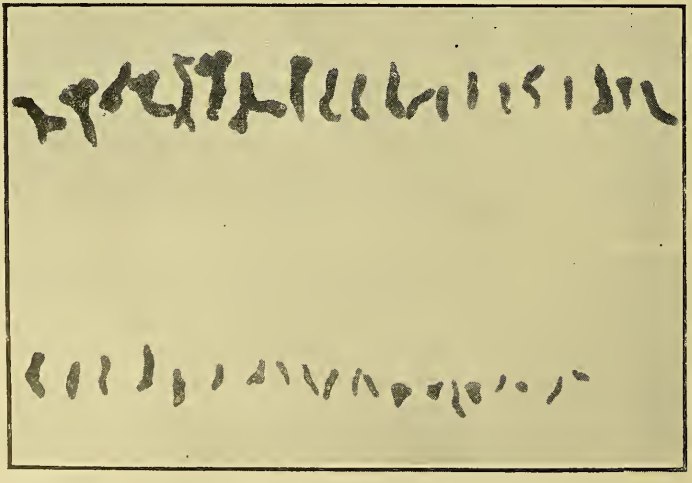

Fig. 1.—Selections of typical nodules from lucerne grown for 86 days in acid sand (above) and alkaline sand (below). (S. Woodward-Smith photo.) × approximately $^4/_3$.

was adjusted to four ranges of reaction by addition of increasing doses of calcium carbonate besides a nutrient mixture of 0·5 gm. KH_2PO_4; 0·5 gm. $CaSO_4$; 0·25 gm. $MgSO_4$; 0·25 gm. NaCl; 0·1 gm. $FeCl_3$; and 3 mgm. of the minor salts. Lucerne and subterranean clover, eight plants per pot, were sown on 31st July and 27th August, 1944, respectively, and harvested after 140 and 108 days.

As shown in Table 2, the net gains of nitrogen by lucerne are not significantly influenced by the reaction, even at pH initially below 5, but the gain per gm. dry nodule is at this reaction significantly lower than at the three other pH-levels. Nodules are still formed at pH (initially) 4·7–4·9, which is below the pH-limit for nodule formation in agar culture (Jensen, 1943), but the numbers of nodules show a marked increase between the pH-levels of 5·4–5·5 and 6·3–6·6. The results thus confirm the earlier observation that the activity of nodules already formed is much less sensitive to acidity than is the formation of new nodules.

TABLE 2.

Nitrogen Fixation by Lucerne and Subterranean Clover at Four Ranges of Reaction.

		Lucerne (31/7/44–18/12/44).				Clover (28/7/44–13/11/44).			
% $CaCO_3$ added		0	0·01	0·04	0·4	0	0·01	0·04	0·4
pH of sand, initial		4·7–4·9	5·4–5·5	6·3–6·6	7·0–7·1	4·5	4·8–4·9	6·0–6·3	6·7–6·8
,, ,, ,, final		4·3–4·5	5·0–5·1	6·9–7·2	7·6–7·7	4·1–4·3	4·5–4·7	6·2–6·4	7·6–7·7
Dry matter, gm. per pot, mean.	Tops ..	6·20	7·22	7·22	8·33	5·35	5·68	5·27	5·17
	Roots ..	5·75	5·05	5·78	6·02	0·94	1·21	1·08	1·03
	Nodules ..	0·148	0·088	0·101	0·106	0·157	0·146	0·152	0·208
Percentage of N in dry matter.	Tops ..	2·68	2·84	2·35	2·78	3·05	3·05	2·61	2·56
	Roots ..	2·32	2·49	2·37	1·38	3·24	2·58	2·92	2·59
	Nodules ..	7·44	6·99	7·20	6·81	7·12	7·83	6·82	6·52
Total N in plants, mgm.		309·9	339·5	313·9	325·3	205·1	215·9	181·9	171·2
Net gain of N, mgm.		278·6	308·2	288·3	299·7	170·2	181·0	156·3	145·6
Do. per gm. dry nodule-substance ..		1939	3539	2874	2846	1112	1236	1010	722
Nodules per plant.	Mean ..	18·4	25·2	57·2	50·4				
	S.E. ..	±2·37	±4·63	±4·47	±5·32				
		Significant difference at P=				Significant difference at P=			
		0·05	0·02	0·01		0·05	0·02	0·01	
Net gain of N		52·1	65·5	75·9		96·4	121·2	140·3	
Do. per gm. dry nodule-substance		692	870	1004		351	441	511	

The net gains of nitrogen in clover, although rather low and irregular, are also unaffected by the reaction, but the gain per gm. dry nodule shows a significant decline at the highest pH-level. The nodules were not counted in this case. The results are hardly altogether conclusive, because the plants suffered badly from attack by mites, but they do confirm the observation that subterranean clover can fix nitrogen in sand of pH 4·1–4·5. No further experiments were undertaken with this sand, for reasons explained below.

Main Series of Experiments with Lucerne.

Experiment No. 1.—Lucerne was sown on 28th February, 1944, eight plants per pot, and harvested after 64, 92 and 120 days. The nutrients consisted of 0·5 gm. KH_2PO_4; 0·1 gm. K_2HPO_4; 0·6 gm. $CaCl_2$; 0·3 gm. $MgSO_4$; 0·03 gm. $FeCl_3$; 3·0 mgm. minor salts; and 0·1% $CaCO_3$ in the alkaline sand. The results in Table 3 show that neither the actual gains of nitrogen nor the gains per gm. dry nodule are significantly different at the two ranges of reaction, even at the final harvest when the pH of acid sand has fallen well below 5. The numbers of nodules are, especially in the early stages, much lower

in the acid sand, but their aggregate weight is practically unaffected by the reaction, and their activity shows no clear-cut change with increasing age.

Experiment No. 2.—The acid hill sand was used, with the following nutrients: 0·4 gm. KH_2PO_4; 0·1 gm. K_2HPO_4; 0·5 gm. $CaSO_4$; 0·25 gm. $MgSO_4$; 0·1 gm. $FeCl_3$; 3·0 mgm. minor salts; and 0·1% $CaCO_3$ in the alkaline sand. Lucerne, eight plants per pot, was sown on 25th August, 1944, and harvested after 60, 85 and 100 days. Table 4 shows the results. The sand without calcium carbonate rapidly becomes strongly acid; the net gains of nitrogen are significantly lower than in the faintly alkaline sand, and the gain per gm. dry nodule shows a very marked reduction at the final harvest. This confirms the former observation (Table 2) that an acidity of pH 4·5–4·8 is too high for

TABLE 3.

Nitrogen Fixation by Lucerne, Experiment No. 1 (28/2/44–27/6/44).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
$-CaCO_3$	64	5·0–5·7	0·55	0·14	0·012	3·29	2·12	8·02	3·4± 0·60
Initial pH	92	5·1–5·5	1·65	0·72	0·055	3·84	2·34	6·72	21·4± 2·97
5·7	120	4·6–4·8	3·99	3·26	0·142	3·72	2·05	8·87	41·8± 5·82
$+CaCO_3$	64	6·9–7·0	0·74	0·25	0·021	4·03	2·20	7·77	21·1± 2·46
Initial pH	92	7·1–7·2	1·68	1·08	0·048	4·53	2·38	6·76	42·7± 3·09
6·7	120	7·0–7·1	3·89	3·29	0·135	4·24	2·20	9·26	127·9±16·3

Summary :

Days.	Total N in Plants, mgm.		Net Gain of N, mgm.			Gain of N, mgm. per gm. Dry Nodule-substance.		
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.
64	22·0	36·9	21·0	29·6	—	1750	1410	—
92	84·0	108·7	76·2	94·9	—	1380	1977	—
120	227·7	249·7	218·9	241·7	—	1541	1790	—

TABLE 4.

Nitrogen Fixation by Lucerne in Hill Sand, Experiment No. 2 (25/8/44–5/12/44).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
$-CaCO_3$	60	4·8–5·0	0·34	0·14	0·012	3·54	2·18	8·04	3·5±0·63
Initial pH	85	4·6–4·8	2·13	1·38	0·078	4·07	2·54	7·39	9·9±1·68
5·1	100	4·5–4·6	3·61	2·72	0·123	2·88	2·36	6·54	17·7±2·79
$+CaCO_3$	60	7·1–7·2	0·83	0·22	0·026	3·76	1·25	7·68	16·1±0·68
Initial pH	85	7·0–7·4	2·95	2·27	0·080	2·85	2·35	8·38	26·4±2·49
7·2	100	7·2–7·5	4·93	3·87	0·098	3·20	2·39	7·20	30·3±3·58

Summary:

Days.	Total N in Plants, mgm.		Net Gain of N, mgm.			Gain of N, mgm. per gm. Dry Nodule-substance.		
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.
60	16·0	35·9	(0)	17·8			712	
85	127·3	144·2	99·0	119·8	+	1298	1506	−
100	175·1	257·7	143·8	232·1	++	1209	2321	+++

maximum nitrogen fixation, but the process still continues. The numbers and weights of nodules show the same behaviour as previously observed; the small but apparently significant increase (7·8 ± 3·26) in numbers between the 85th and the 100th day at pH 4·5–4·8 is noteworthy.

This sand had unfortunately a high content of metabolizable nitrogen and produced up to 10 p.p.m. NO_3–N, which involved an undesirably large correction for nitrogen taken from the medium (hence no gain of nitrogen in acid sand after 60 days). It was therefore not used any more except for one experiment with subterranean clover (Table 9) conducted together with the present.

Experiment No. 3.—This comprised two series, with free and combined nitrogen. The basal nutrients were 0·5 gm. KH_2PO_4; 0·5 gm. $CaCl_2$; 0·25 gm. $MgSO_4$; 0·25 gm. NaCl; 0·1 gm. $FeCl_3$; 3·0 mgm. of the minor salts; and 0·2% $CaCO_3$ in the alkaline sand. Combined nitrogen was provided as 120 mgm. as $NaNO_3$ per pot at start, and 3 × 60 mgm. as $(NH_4)_2SO_4$ after 57, 77 and 91 days; this mixture was chosen in order to minimize the pH-changes due to preferential assimilation of the nitrate- and ammonium-ions. Lucerne was sown on 23rd February, 1945, and harvested after 56, 76, 91 and 105 days, six plants per pot.

TABLE 5.

Nitrogen Fixation and Uptake of Combined Nitrogen by Lucerne, Experiment No. 3 (23/2/45–5/6/45).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
Free N	56	6·0–6·2	1·07	0·38	0·042	3·59	1·94	6·82	9·6±1·53
$-CaCO_3$	76	5·1–5·6	3·51	2·32	0·106	3·84	2·46	7·85	17·5±1·02
Initial pH	91	5·1–5·4	4·35	3·73	0·170	3·56	2·12	7·84	42·0±5·43
5·4–5·5	105	4·9 5·4	5·28	5·45	0·178	2·92	2·01	6·85	46·0±4·46
Free N	56	7·3–7·4	1·42	0·61	0·047	3·60	2·38	6·70	21·4±1·61
$+CaCO_3$	76	7·6–7·8	3·11	1·73	0·095	3·20	2·23	8·02	25·6±2·41
Initial pH	91	7·2–7·3	4·55	3·76	0·148	3·89	1·83	6·92	39·3±3·72
6·8–7·0	105	7·2–7·3	5·93	6·08	0·176	3·40	2·21	6·79	53·0±6·99
Comb. N	56	6·8–7·0	2·27	0·98	0·018	3·60	2·22	6·01	17·5±2·62
$-CaCO_3$	76	5·6–6·0	4·31	3·73	0·095	2·75	2·10	7·25	38·8±3·09
Initial pH	91	4·7–5·1	5·24	4·93	0·094	3·47	2·28	7·75	32·0±3·36
5·4–5·5	105	4·9–5·0	7·17	6·78	0·139	3·28	2·08	7·19	37·3±3·97
Comb. N	56	8·1–8·2	2·17	0·86	0·016	3·55	2·30	7·10	17·2±2·99
$+CaCO_3$	76	7·6–7·8	4·69	3·62	0·097	2·95	1·94	8·84	29·8±2·89
Initial pH	91	7·0–7·4	5·15	4·84	0·084	3·30	2·08	6·72	50·6±7·32
6·8–7·0	105	7·2–7·3	6·52	6·77	0·100	3·12	2·08	7·04	44·3±3·83

Summary: Free-nitrogen series.

Days.	Total N in Plants, mgm.		Net Gain of N, mgm.			Gain of N, mgm. per gm. Dry Nodule-substance.		
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.
56	48·9	67·7	46·3	62·2	−	1140	1345	−
76	202·4	145·3	198·1	140·8	+	1857	1612	−
91	249·7	255·9	243·8	251·8	−	1443	1706	+++
105	276·0	347·5	271·2	344·0	−	1516	1967	+

Summary: Combined-nitrogen series.

Days.	Total N in Plants, mgm.		Significance of Difference.	Net Gain of N, mgm. (assumed).	
	$-CaCO_3$.	$+CaCO_3$.		$-CaCO_3$.	$+CaCO_3$.
56	104·5	97·9	−	(0)	(0)
76	203·5	217·2	−	(29·1)	(47·2)
91	301·6	273·4	−	(55·7)	(29·3)
105	385·2	351·3	−	(80·4)	(47·8)

Relative Numbers and Weights of Nodules.		Acid Sand after Days.				Alkaline Sand after Days.			
		56	76	91	105	56	76	91	105
Nodules, number per gm. dry root.	Free N ..	165	45	74	58	99	93	65	59
	Comb. N ..	103	63	41	35	126	46	68	39
Weight of nodules in % of total dry matter.	Free N ..	2·78	1·76	2·07	1·63	2·22	1·79	1·75	1·46
	Comb. N ..	0·53	1·16	0·91	0·98	0·44	1·13	0·83	0·73

The results in Table 5 show that the growth with free nitrogen is somewhat irregular, and the reaction has as a whole no significant influence on the net gains of nitrogen, except after 77 days, when the gain is higher at acid reaction. The gains per gm. dry nodule at the two last stages show significantly lower values at pH 4·9–5·4 than at pH 7·2–7·3, although the difference is not very conspicuous. The numbers of nodules are in this case not significantly different at the two reactions after 91 and 105 days; a possible explanation may be that extensive infection of the roots has taken place during the first 56 days when the sand was only faintly acid, the nodules only later becoming visible to the naked eye.

The plants with combined nitrogen grew more rapidly than with free nitrogen. At no stage does the reaction significantly affect the uptake of nitrogen, but unfortunately the pH-values are very inconstant; after 56 days the sodium nitrate had almost neutralized the acid sand and rendered the sand with calcium carbonate strongly alkaline. Except at the first harvest the crops at both reactions contain more nitrogen than was added in combined form (180, 240 and 300 mgm.), and small amounts of nitrate and ammonia were found in the sand after harvest; thus the plants appear to have fixed some nitrogen in the presence of excess available nitrogen in the growth substrate, as

also observed by Giöbel (1926). An estimate of the gain of nitrogen in these pots was made by subtracting the nitrogen added to the sand, plus the mineral nitrogen produced in unplanted control sand, from the nitrogen content of the plants plus the mineral nitrogen found in the sand after harvest. These estimates are of course only tentative, *inter alia*, because of the possibility that some mineral nitrogen may disappear through microbial processes in the sand, and especially the plant rhizospheres. The gains of nitrogen per gm. dry nodule seem to range between 300 and 600 mgm., or roughly one-sixth to one-third of the corresponding values in the free-nitrogen series. The provision of combined nitrogen in a quantity comparable to the fixation by plants in nitrogen-free sand in the same time has thus strongly reduced the nitrogen-fixing efficiency of the nodule tissue, without, however, suppressing it completely. The numbers of nodules are little influenced by the reaction and are not significantly lower, after 56 and 76 days at acid reaction even higher, than in the free-nitrogen series. When the more rapid growth with combined nitrogen is taken into consideration and the number of nodules expressed on the basis of dry root weight, the numbers appear somewhat, but not constantly or markedly, lower in the presence of combined nitrogen (cf. Thornton and Nicol, 1936, and many earlier workers quoted by Fred et al., 1932). A far more striking effect of the combined nitrogen is the great decrease in the average size of the nodules, as also found by Giöbel (1926) and Thornton and Nicol (1936), and consequently in the weight of the nodule-fraction in proportion to the whole plant; this is particularly noticeable at the first harvest when the excess of combined nitrogen was highest and no nitrogen had yet been fixed.

Experiment No. 4.—Combined nitrogen was given as ammonium nitrate, in order to avoid drastic pH-changes. The basal nutrients were 0·4 gm. KH_2PO_4; 0·4 gm. $CaCl_2$; 0·2 gm. $MgSO_4$; 0·2 gm. NaCl; 0·08 gm. $FeCl_3$; 1·2 mgm. of the minor salts; and 0·1% $CaCO_3$ in the alkaline sand. The pots in the combined-nitrogen series received at first 24, later 48 mgm. nitrogen per week in two doses, so as to imitate the conditions in a vigorously nitrifying soil. Lucerne, six plants per pot, was sown on 20th July, 1945, and harvested after 35, 68, 88 and 102 days, when the combined-nitrogen pots had received 60, 192, 276 and 372 mgm. nitrogen respectively. Table 6 gives the results.

TABLE 6.

Nitrogen Fixation and Uptake of Combined Nitrogen by Lucerne, Experiment No. 4 (20/7/45–30/10/45).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
Free N,	35	6·1–6·2	—	—	—	—	—	—	5·5±1·17
—$CaCO_3$.	68	5·8–6·2	0·72	0·42	0·050	3·62	2·38	6·89	12·1±1·10
Initial pH,	88	5·4–5·6	2·10	1·42	0·083	3·23	2·26	6·68	11·4±1·09
5·1–5·2	102	5·3–5·6	3·61	2·89	0·164	3·11	2·23	6·57	12·8±0·99
Free N,	35	7·0	—	—	—	—	—	—	8·5±0·81
+$CaCO_3$.	68	7·2–7·5	1·11	0·62	0·064	3·42	2·00	6·56	33·8±3·71
Initial pH,	88	7·2–7·5	2·92	2·14	0·103	3·14	2·20	7·05	48·9±4·32
6·8–6·9	102	7·3–7·4	4·31	3·92	0·169	2·86	2·18	6·83	51·5±6·47
Comb. N,	35	5·7–5·8	—	—	—	—	—	—	0·3±0·55
—$CaCO_3$.	68	5·1–5·2	1·42	0·79	0·011	3·82	2·64	6·07	9·9±1·52
Initial pH,	88	4·9–5·5	3·59	2·42	0·039	3·37	2·44	5·78	7·1±1·10
5·1–5·2	102	4·7–5·0	5·65	5·54	0·054	2·89	2·31	4·95	6·6±1·26
Comb. N,	35	6·7–6·8	—	—	—	—	—	—	4·9±0·76
+$CaCO_3$.	68	7·5–7·7	1·95	0·92	0·011	3·62	2·57	6·00	32·0±7·14
Initial pH,	88	7·6–7·8	4·42	3·14	0·035	3·17	2·26	6·80	38·3±4·15
7·5–8·0	102	7·5–8·0	6·47	5·92	0·035	2·73	2·25	6·00	27·7±3·38

Summary: Free-nitrogen series.

Days.	Total N in Plants, mgm.		Net Gain of N, mgm.			Gain of N, mgm. per gm. Dry Nodule-substance.		
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.
35	1·7	2·9	(0)	(0)	—	(0)	(0)	—
68	39·6	54·4	37·2	51·4	—	743	808	—
88	105·4	145·5	103·0	142·5	+++	1241	1394	—
102	187·3	220·0	179·8	214·9	—	1097	1284	—

Summary: Combined-nitrogen series.

Days.	Total N in Plants, mgm.		Significance of Difference.
	$-CaCO_3$.	$+CaCO_3$.	
35	2·0	2·7	—
68	75·3	94·7	—
88	182·4	213·4	—
102	293·3	311·8	—

Relative Numbers and Weights of Nodules.		$-CaCO_3$, after Days.			$+CaCO_3$, after Days.		
		68	88	102	68	88	102
Number of nodules per gm. dry root.	Free N ..	184	49	27	326	144	96
	Comb. N ..	71	17	7	201	74	28
Weight of nodules in % of total dry matter.	Free N ..	4·23	2·32	2·37	3·55	2·00	2·00
	Comb. N ..	0·50	0·61	0·47	0·36	0·44	0·26

The growth was at first very slow, owing to a period of cold weather. Addition of nitrogen was therefore interrupted for 2½ weeks, and only total nitrogen and numbers of nodules were determined after 35 days. In the free-nitrogen series the net gain of nitrogen is after 88 days significantly lower at acid reaction, a rather unexpected result in view of the only moderately high acidity. The gains per gm. dry nodule are not significantly different, but upon the whole lower than in any other experiment. The numbers of nodules in acid sand, too, appear unusually low, which may perhaps explain the small crop yield after 88 days.

The combined-nitrogen series shows better growth, but the added nitrogen was in no case used up, and no nitrogen appears to have been fixed. The total nitrogen content of the plants is not significantly influenced by the reaction, although the acidity of the acid sand increased considerably. The actual numbers of nodules are only at the last harvest markedly lower than in the corresponding free-nitrogen pots. On the other hand the provision of combined nitrogen has greatly reduced the numbers of nodules per gm. root, especially in the acid sand. An even more conspicuous effect is seen on the proportional weight of the nodules, which is only one-tenth to one-fourth of the

corresponding figures in the free-nitrogen series. It is noteworthy that this effect of the combined nitrogen is much more pronounced in the present experiment, where a considerable excess of available nitrogen was left in the medium, than in the previous one (Table 5).

Experiment No. 5.—This was designed like the previous one, except that 1·5 mgm. of the minor salts was given and the combined-nitrogen pots received first 15 and later 30 mgm. nitrogen per week in two doses. Lucerne was sown on 31st August, 1945, and harvested after 59, 74, 88 and 102 days, when the combined-nitrogen series had received 180, 240, 300 and 360 mgm. nitrogen per pot. Table 7 shows the results.

The plants grew more vigorously than in the previous experiment, especially in the free-nitrogen series where the reaction of the sand without calcium carbonate is only moderately acid. The net gains of nitrogen are not significantly different, except perhaps at the first harvest, and the gains per gm. dry nodule are barely significantly higher in neutral than in acid sand at the first and third harvest, and generally on a high level. The nodules develop slowly in the acid sand, but at the last harvest their numbers are as high as at pH 7·0–7·5.

TABLE 7.

Nitrogen Fixation and Uptake of Combined Nitrogen by Lucerne (31/8/45–11/12/45).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
Free N,	59	5·4–5·9	1·56	0·59	0·059	3·19	2·34	6·41	10·0±1·46
−$CaCO_3$.	74	5·3–5·6	3·47	1·93	0·091	3·30	2·46	7·36	15·7±2·01
Initial pH,	88	5·1–5·6	4·82	3·21	0·136	3·23	2·40	7·18	28·4±3·14
5·4–5·7	102	6·0–6·1	7·01	5·88	0·172	3·15	2·44	7·18	46·2±4·21
Free N,	59	7·3–7·7	2·16	0·85	0·055	3·52	2·26	6·91	26·8±2·66
+$CaCO_3$.	74	7·0–7·3	3·83	2·16	0·082	3·21	2·39	6·69	40·9±3·66
Initial pH,	88	7·3–7·6	5·18	3·45	0·095	2·97	2·27	8·14	41·1±3·91
7·1–7·3	102	7·0–7·5	7·33	4·68	0·129	2·98	1·99	6·93	50·7±5·66
Comb. N,	59	4·9–5·5	2·16	0·95	0·013	3·68	2·76	7·08	4·2±0·85
−$CaCO_3$.	74	4·9–5·0	4·75	3·34	0·051	3·00	2·40	5·54	6·9±1·26
Initial pH,	88	4·5–5·0	5·57	3·87	0·028	3·04	2·58	5·03	6·2±0·98
5·4–5·7	102	4·9–5·0	7·90	6·06	0·061	2·75	2·27	4·31	4·6±0·88
Comb. N,	59	7·1–7·7	3·56	1·86	0·033	3·41	2·25	7·07	30·6±2·81
+$CaCO_3$.	74	7·1–7·2	5·30	3·53	0·037	3·06	2·40	6·02	28·8±2·55
Initial pH,	88	7·5–7·6	6·60	5·59	0·040	2·86	2·34	5·98	28·5±3·42
7·1–7·3	102	7·4–7·6	8·25	7·09	0·042	2·64	2·22	5·95	30·4±2·29

Summary: Free-nitrogen series.

Days.	Total N in Plants, mgm.		Net Gain of M, mgm.			Gain of N, mgm. per gm. Dry Nodule-substance.		
	−$CaCO_3$.	+$CaCO_3$.	−$CaCO_3$.	+$CaCO_3$.	Significance of Difference.	−$CaCO_3$.	+$CaCO_3$.	Significance of Difference.
59	67·0	99·0	59·9	89·5	+	1073	1821	+
74	168·5	180·2	161·4	170·7	−	1861	2141	−
88	242·3	240·0	229·8	224·5	−	1733	2407	+
102	376·9	320·2	359·0	306·8	−	2083	2335	−

Summary: Combined-nitrogen series.

Days.	Total N in Plants, mgm.			Net Gain of N, mgm.		
	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.	$-CaCO_3$.	$+CaCO_3$.	
59	101·9	161·2	+++	(0)	(0)	
74	224·1	252·2	—	(0)	(26·8)	Gain of nitrogen in one pot after 74 days and in two after 88 and 102 days.
88	272·0	321·7	—	(0)	(31·0)	
102	357·9	377·7	—	(0)	(25·5)	

Relative Numbers and Weights of Nodules.		$-CaCO_3$, after Days.				$+CaCO_3$, after Days.			
		59	74	88	102	59	74	88	102
Nodules, number per gm. dry roots.	Free N ..	101	52	53	48	189	114	71	66
	Comb. N	26	12	10	5	92	48	30	26
Weight of nodules in % of total dry matter.	Free N ..	2·75	1·63	1·70	1·32	1·73	1·36	1·13	1·08
	Comb. N	0·57	0·63	0·29	0·38	0·54	0·37	0·40	0·27

The growth with combined nitrogen is at first somewhat better than with free nitrogen, but the difference disappears at the second harvest, and the uptake of nitrogen is only at the first harvest significantly lower from the acid sand, in spite of strong increase in acidity during the growth. In a few of these pots all the added nitrogen was used up and some extra nitrogen fixed, but as in Experiment No. 3, the gain per gm. nodule is very low (320–770 mgm.) in comparison with the figures in the free-nitrogen series. The numbers of nodules are greatly depressed by combined nitrogen in acid but less so in alkaline sand; the pH-values of the acid sand, however, are considerably lower in the combined-nitrogen than in the free-nitrogen series, and the decrease in nodule numbers may be as much due to the higher acidity as to the nitrogen *per se*. The decrease in numbers of nodules per gm. dry root and in the proportional weight of the nodules in the presence of combined nitrogen is again very marked.

Some determinations of residual ammonia and nitrate in the sand (mixed from replicate pots) were made in order to detect whether significant quantities of mineral nitrogen disappear otherwise than through uptake by the plants. The following approximate balance-sheet of nitrogen was found:

	Acid Sand, Days.			Alkaline Sand, Days.		
	74	88	102	74	88	102
Mgm. mineral N added per pot ..	240·0	300·0	360·0	240·0	300·0	360·0
Do. formed in unplanted sand ..	6·3	11·7	17·1	8·7	10·7	12·6
Sum	246·3	311·7	377·1	248·7	310·7	372·6
Do. found in sand after harvest ..	30·0	24·5	18·3	21·3	(0)	11·4
Disappeared from sand	216·3	287·2	358·8	227·4	310·7	361·2
Recovered in plants (total less seed-N)	223·3	271·2	357·1	251·3	320·9	376·9

Except in alkaline sand after 74 days the nitrogen contents of the plants correspond closely to the estimated amounts of disappeared mineral nitrogen, and there is no indication that significant proportions of the added nitrogen have been consumed by other processes such as denitrification or synthesis of microbial protoplasm in the sand or the rhizospheres.

Main Series of Experiments with Subterranean Clover.

These experiments were conducted as parallels to the experiments with lucerne, with the same nutrient treatments but different periods of growth and sometimes different sowing date.

Experiment No. 1 (*cf. lucerne experiment No. 1, Table 3*).—Clover was sown on 8th March, 1944, eight plants per pot, and harvested after 62, 92 and 125 days. The results are shown in Table 8. The sand without calcium carbonate became strongly acid during growth, and the final net gain of nitrogen appears lower than at neutral reaction, but wide variation between the replicates renders the difference non-significant. The numbers of nodules and the gains of nitrogen per gm. dry nodule are the same at both reactions, except that the numbers appear slightly higher after 62 days in neutral sand. The reaction showed a conspicuous effect on the appearance of the root nodules both in this and other experiments with subterranean clover; in acid sand the nodules were typically of a grapeseed-like shape and of more uniform size and distribution than in alkaline sand, where generally a limited number of big lobate nodules were found clustered round the top of the main root, while many small nodules were scattered over the rest of the root system.

Experiment No. 2 (*cf. lucerne experiment No. 2, Table 4*).—Clover was sown on 4th August, 1944, eight plants per pot, and harvested after 73, 94 and 115 days. As seen from Table 9, the growth in this sand has been strongly inhibited by calcium carbonate. Although the reaction is only moderately alkaline, the final net gain of nitrogen is little more than one-half of the gain in acid sand where vigorous nitrogen fixation has, as in Table 2, taken place during the last period at pH 4·2–4·8. The gains per gm. dry nodule are not, on the other hand, influenced by the wide difference in reaction. The actual numbers of nodules are lower in alkaline sand, except perhaps at the first harvest, but calculation of the numbers per gm. dry root show both in this and the previous experiment little effect of the reaction, again excepting the first stage of growth:—

	Experiment No. 1 (Table 8).			Experiment No. 2 (Table 9).		
Days	62	90	125	73	94	115
Nodules per gm. root:						
In acid sand	321	353	265	163	191	212
In alkaline sand	530	368	193	221	229	211

It thus appears that the infective power of *Rhizobium trifolii* towards *Trifolium subterraneum* is not lower at pH 4·7–5·0 than at pH 7·0–7·6, a result which agrees with previous observations in agar culture (Jensen, 1943). The lower numbers of nodules in the second experiment may be due to the higher content of metabolizable nitrogen in the hill sand.

Experiment No. 3 (*cf. lucerne experiment No. 3, Table 5*).—Sowing took place on 25th March, 1945; eight plants per pot were harvested after 75, 96, 116 and 130 days. The early growth was slow, and the combined-nitrogen pots were therefore given only two doses of ammonium sulphate, so that each pot received 240 mgm. nitrogen.

TABLE 8.

Nitrogen Fixation by Subterranean Clover, Experiment No. 1 (8/3/44–11/7/44).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
$-CaCO_3$.	62	4·8–4·9	0·66	0·096	0·033	2·82	0·54	7·42	30·8±1·87
Initial pH,	90	4·9–5·0	1·17	0·18	0·069	3·34	2·19	7·55	63·5±2·95
5·7	125	4·8–5·0	3·14	0·64	0·186	3·59	2·67	10·07	168·5±9·69
$+CaCO_3$.	62	6·9–7·2	0·61	0·081	0·023	3·38	0·42	7·68	42·9±3·77
Initial pH,	90	7·0–7·2	1·49	0·21	0·071	2·76	2·52	8·02	77·3±7·43
6·8	125	7·1–7·2	4·69	0·93	0·197	3·43	2·21	8·47	179·9±15·1

Summary :

Days.	Total N in Plants, mgm.		Neg Gain of N, mgm.			Gain of N, mgm. per gm. Dry Nodule-substance.		
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.	$-CaCO_3$.	$+CaCO_3$.	Significance of Difference.
62	21·5	22·9	16·9	12·0	—	519	528	—
90	48·2	51·9	36·8	37·7	—	539	532	—
125	148·6	197·9	136·2	186·1	—	732	944	—

TABLE 9.

Nitrogen Fixation by Subterranean Clover in Hill Sand, Experiment No. 2 (4/8/44–27/11/44).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
$-CaCO_3$.	73	5·1–5·6	1·60	0·36	0·063	2·78	2·35	6·46	58·8±6·99
Initial pH,	94	4·7–4·8	3·53	0·73	0·155	3·04	2·58	6·56	139·1±13·0
5·3–5·4	115	4·2–4·8	5·46	0·79	0·137	3·00	2·17	5·48	167·4±13·2
$+CaCO_3$.	73	7·2–7·6	1·27	0·20	0·045	2·67	2·28	6·41	44·2±3·18
Initial pH,	94	7·4–7·5	1·97	0·31	0·080	2·82	2·05	7·10	71·1±6·31
7·3–7·6	115	7·3–7·6	4·19	0·46	0·079	2·35	2·30	5·61	97·1±5·44

Summary :

Days.	Total N in Plants, mgm.		Net Gain of N, mgm.		Significance of Difference.	Gain of N, mgm. per gm. Dry Nodule-substance.		Significance of Difference.
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.		$-CaCO_3$.	$+CaCO_3$.	
73	57·0	40·9	28·4	14·7	—	440	419	—
94	138·5	74·0	103·6	43·3	—	665	532	—
115	188·3	113·2	153·4	87 6	+++	1152	1108	—

The results in Table 10 show considerable decrease in acidity of the acid sand at the two first harvests of the free-nitrogen series. The differences in net gain of nitrogen are not significant, but the gains per gm. dry nodule show a certain irregularity, being higher at acid reaction after 130 days, but at alkaline reaction after 96 and 116 days, and the means of the four values at each reaction are almost identical (935 and 925 mgm.). Apart from the first harvest the numbers of nodules are consistently higher in the acid sand, and this is further accentuated when the numbers are expressed on the basis of dry root weight. Apparently a reaction of pH 5·6–6·0 is very favourable for root infection; this reaction also appears optimal for growth of *Rhizobium trifolii* in pure culture (Snieszko, 1928; Jensen, 1942).

The combined-nitrogen series shows only slightly higher yields of dry matter and nitrogen than the free-nitrogen series. The total yields of nitrogen are not significantly different except at the first harvest, when the acid sand has become almost neutralized by the sodium nitrate. After 130 days all added nitrogen is used up and small extra amounts fixed in some of the pots; these gains corresponded to only 120–190 mgm. per gm. dry nodule. The numbers of nodules in acid sand are not much different from those in the corresponding series with free nitrogen, but in acid sand the numbers are quite low, particularly at the two first harvests. On a basis of dry root weight the

TABLE 10.

Nitrogen Fixation and Uptake of Combined Nitrogen by Subterranean Clover, Experiment No. 3.

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
Free N,	75	6·1–6·2	0·70	0·10	0·022	3·95	2·51	8·71	14·6± 1·71
$-CaCO_3$.	96	5·9–6·0	1·59	0·25	0·069	3·09	2·27	7·57	38·5± 7·52
Initial pH,	116	5·6–5·7	3·45	0·70	0·186	3·01	2·25	8·32	103·5±19·6
4·9–5·0	130	4·9–5·0	6·27	1·02	0·176	3·27	2·63	6·59	90·0±15·3
Free N,	75	7·2–7·3	0·88	0·16	0·035	3·20	2·22	8·34	14·9± 2·61
$+CaCO_3$.	96	7·3–7·5	1·45	0·22	0·048	2·95	2·16	8·78	18·1± 3·30
Initial pH,	116	7·3–7·4	3·45	0·59	0·138	3·63	2·22	7·90	40·6± 9·69
6·8–6·9	130	7·2–7·3	6·23	1·28	0·224	2·88	1·09	6·53	61·4±10·9
Comb. N,	75	6·3–6·9	1·21	0·21	0·007	3·64	2·93	9·70	22·8± 6·54
$-CaCO_3$.	96	6·4–6·5	1·96	0·33	0·014	3·33	2·57	9·65	40·5±11·6
Initial pH,	116	5·6–5·8	5·12	1·09	0·114	2·96	2·10	7·55	96·9±15·6
4·9–5·0	130	4·8–5·3	6·75	1·50	0·135	2·93	2·71	6·56	111·5±22·1
Comb. N,	75	7·0–7·2	0·64	0·10	0·003	4·21	2·67	—	7·2±1·24
$+CaCO_3$.	96	7·1–7·2	1·47	0·21	0·004	3·36	2·61	7·98	8·7± 1·55
Initial pH,	116	7·3	4·17	0·93	0·043	3·15	2·45	7·09	42·5±10·4
6·8–6·9	130	7·2	7·19	1·63	0·113	2·93	2·38	7·47	58·7±14·4

Summary: Free-nitrogen series.

Days.	Total N in Plants, mgm.		Net Gain of N, mgm.		Significance of Difference.	Gain of N, mgm. per gm. Dry Nodule-substance.		Significance of Difference.
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.		$-CaCO_3$.	$+CaCO_3$.	
75	32·2	34·1	24·1	25·0	—	1019	852	—
96	60·7	51·8	51·6	43·9	—	735	904	+
116	134·8	149·4	126·2	142·0	—	682	1050	++
130	240·0	208·2	230·3	201·9	—	1304	893	++

Summary: Combined-nitrogen series.

Days.	Total N in Plants, mgm.		Significance of Difference.	Net Gain of N, mgm.		
	$-CaCO_3$.	$+CaCO_3$.		$-CaCO_3$.	$+CaCO_3$.	
75	50·4	29·8	+	(0)	(0)	Gain of nitrogen after 130 days in one pot $-CaCO_3$ and in two $+CaCO_3$.
96	74·7	56·9	–	(0)	(0)	
116	182·9	156·8	–	(0)	(0)	
130	248·9	252·4	–	(18·9)	(25·1)	

Relative Numbers and Weights of Nodules.		$-CaCO_3$, after Days.				$+CaCO_3$, after Days.			
		75	96	116	130	75	96	116	130
Number of nodules per gm. dry root.	Free N ..	1424	1239	1196	716	724	650	508	375
	Comb. N ..	844	1224	736	608	591	331	368	270
Weight of nodules in % of total dry matter.	Free N ..	2·71	3·40	4·25	2·35	3·22	2·81	3·27	2·92
	Comb. N ..	0·49	0·60	1·73	1·59	0·41	0·27	0·76	1·26

numbers of nodules are greatly diminished by alkaline reaction, and much less so by the provision of combined nitrogen. The proportional weight of the nodules is on the other hand not markedly influenced by the reaction, but is greatly depressed by combined nitrogen; it is noteworthy that the weight increases towards the end of the growth period when the sand has been exhausted of combined nitrogen and fixation begins to take place.

Experiment No. 4 (*cf. lucerne experiment No. 4, Table 6*).—Clover was sown on 1st August, 1945; six plants were left per pot and harvested after 63, 82, 96 and 110 days. The combined-nitrogen pots received at first 2 × 12 and later 2 × 24 mgm. nitrogen as ammonium nitrate per week, starting one week after sowing, so that the total doses at the four harvests amounted to 180, 276, 372 and 468 mgm. per pot. Growth was more vigorous in this than in any other experiment. Table 11 gives the results.

The acid sand in the free-nitrogen series shows rapidly increasing acidity. The net gains of nitrogen are all significantly lower than in the alkaline sand, but very strong fixation still takes place between the 82nd and 96th day, during which interval the pH-level falls from 5·0–5·2 to 4·3–4·7. The results of the final harvest are of limited significance because the growth increased very little during the last stage, and the roots showed signs of beginning decay at both acid and alkaline reaction.

The gain of nitrogen per gm. dry nodule is on the other hand at no stage significantly influenced by the reaction, but the figures are lower and the proportional weights of the nodules higher than in any other experiment. The numbers of nodules are, except at the first harvest, two to three times as high in acid as in alkaline sand, and the differences become even more pronounced when expressed in terms of nodules per gm. dry root. Results like these illustrate particularly well how little correlation there is between the actual gain of nitrogen and the number of nodules responsible for it.

The growth in the combined-nitrogen series, especially in the early stages, is even more vigorous than with free nitrogen, which suggests that favourable light and temperature conditions have given rise to a rate of photosynthesis too high for optimum nitrogen fixation (cf. Wilson, 1940); possibly the unusually high proportional weight of

the nodule-fraction might be explained through this. The acid sand shows pH-values as low as in the free-nitrogen series, but the nitrogen content of the plants is at no stage significantly lower, but rather higher, than in the alkaline sand. After 96 days the added nitrogen was used up in all the acid and one of the alkaline pots, and small amounts of nitrogen were fixed (110–210 mgm. per gm. dry nodule, against 550–600 mgm. in the free-nitrogen series after 96 days); this is accompanied by a notable increase in the proportional weight of the nodules, as in the previous experiment (Table 10). During the final stage very little nitrogen was taken up from the alkaline and none from the acid sand; the apparent decrease in nitrogen content is not significant. The roots showed obvious signs of decay after 110 days, and some nodules were probably lost. The actual numbers of nodules, in comparison with the free-nitrogen series, are only depressed by combined nitrogen at the two last harvests in acid sand; the numbers per gm. dry root show a large decrease at all stages and both reactions, and the same applies to the proportional weight of the nodule-fraction.

TABLE 11.

Nitrogen Fixation and Uptake of Combined Nitrogen by Subterranean Clover, Experiment No. 4 (1/8/45–19/11/45).

Treatment.	Days.	pH of Sand.	Dry Matter, gm.			% N in Dry Matter.			Nodules per Plant, Mean and S.E.
			Tops.	Roots.	Nodules.	Tops.	Roots.	Nodules.	
Free N,	63	5·1–5·2	1·06	0·28	0·095	2·87	2·15	7·33	55·6± 3·73
$-CaCO_3$.	82	5·0–5·2	2·30	0·58	0·156	2·88	2·33	6·73	123·3± 7·76
Initial pH,	96	4·3–4·7	5·82	1·55	0·323	2·53	2·36	6·24	255·8±16·5
5·8	110	4·2–4·4	6·82	1·73	0·375	2·47	2·13	5·42	241·4±14·2
Free N,	63	7·1–7·4	1·47	0·46	0·135	2·88	2·10	7·90	56·2± 2·61
$+CaCO_3$.	82	7·3–7·5	3·76	1·01	0·228	2·80	2·13	6·93	66·1± 3·89
Initial pH,	96	7·1–7·2	8·71	2·08	0·476	2·44	2·19	6·52	80·5± 3·18
7·0	110	7·5–7·6	10·74	2·16	0·487	2·40	2·03	5·48	101·8± 8·43
Comb. N,	63	5·1–5·2	3·70	1·24	0·051	2·81	2·74	6·88	117·8±10·4
$-CaCO_3$.	82	5·0–5·9	7·04	2·06	0·150	2·74	2·55	6·98	120·2± 8·38
Initial pH,	96	4·3–4·7	10·62	3·75	0·241	2·73	2·79	6·19	131·9± 7·75
5·8	110	4·2–4·4	11·62	3·51	0·163	2·52	2·44	5·62	115·9±10·8
Comb. N,	63	7·5–7·6	2·80	0·96	0·020	3·17	2·82	6·03	75·6± 9·46
$+CaCO_3$.	82	7·4–7·7	5·80	1·96	0·052	2·75	2·62	6·48	73·3± 5·57
Initial pH,	96	7·3–7·5	10·18	3·35	0·131	2·67	2·63	7·18	72·8± 3·21
7·0	110	7·2–7·5	12·81	3·88	0·195	2·42	2·13	6·00	93·0± 7·65

Summary: Free-nitrogen series.

Days.	N in Plants, mgm.		Net Gain of N, mgm.		Significance of Difference.	Gain of N, mgm. per gm. Dry Nodule-substance.		Significance of Difference.
	$-CaCO_3$.	$+CaCO_3$.	$-CaCO_3$.	$+CaCO_3$.		$-CaCO_3$.	$+CaCO_3$.	
63	43·6	62·7	34·2	56·0	+	361	414	–
82	90·3	142·5	80·9	135·8	+++	518	597	–
96	205·1	289·0	196·1	281·7	+++	552	602	–
110	226·0	328·3	216·9	320·4	+++	587	632	–

Summary: Combined-nitrogen series.

Days.	N in Plants, mgm.		Significance of Difference.	Net Gain of N, mgm.		
	$-CaCO_3$.	$+CaCO_3$.		$-CaCO_3$.	$+CaCO_3$.	
63	146·4	116·8	−			Gain after 96 days in alkaline sand in one pot only.
82	257·2	214·4	++			
96	410·3	369·2	−	28·0	(44·4)	
110	386·9	404·3	−			

Relative Numbers and Weights of Nodules.		$-CaCO_3$, after Days.				$+CaCO_3$, after Days.			
		63	82	96	110	63	82	96	110
Nodules, number per gm. dry root.	Free N ..	1191	1263	1011	837	744	398	232	280
	Comb. N	567	342	181	191	436	224	133	155
Weight of nodules in % of total dry matter.	Free N ..	6·65	5·14	4·61	4·15	6·63	4·58	4·23	3·64
	Comb. N	0·90	1·23	1·65	1·03	0·51	0·66	0·93	1·15

The Nitrogen-fixing Efficiency of Root Nodule Tissue in Lucerne and Subterranean Clover.

From the data of the preceding experiments with lucerne and clover grown with free nitrogen we may now calculate the rates of nitrogen fixation by the nodule tissue at successive stages of growth, by the formula of Bond (1936):

$$E = \frac{I}{t \times (w_1 + w_2)/2}$$

where E represents the efficiency of the nodules as fixation of nitrogen in mgm. per gm. dry nodule-substance per day, I the increase in net gain of nitrogen per pot in t days, and w_1 and w_2 the dry weight of the nodules at the beginning and the end of the period.

The figures for lucerne are shown in Table 12, together with the ratios between the amount of nitrogen fixed per day and the mean nitrogen content of the nodules at the beginning and the end of the period. These figures thus express the efficiency of fixation on the basis of nodule nitrogen (E_n) instead of total dry matter ($E_{d.m.}$). The table also contains the rates of transfer of fixed nitrogen from the nodules to the rest of the plant; this is expressed as net gain of top and root nitrogen in per cent. of total net gain, it being assumed that all nitrogen in the nodules results from fixation and not from the small uptake of combined nitrogen.

During the first period of growth the efficiency cannot be determined accurately because we do not know how many days elapse before the nodules begin to function (probably some 10 to 20 days, to judge from the appearance of the first true leaves, which coincides approximately with the beginning of fixation [Fred *et al.*, 1932]); these figures thus represent minimum values. The subsequent rates of fixation vary between 11 and 103 mgm. nitrogen per gm. dry nodule per day. Only in three instances (Experiment No. 2 at pH 4·5–4·8 and Experiment No. 3 at pH 4·9–5·7) is there a real indication of reduced nodular efficiency at acid reaction, and in Experiment No. 3 the decline in the 76–91 days period even occurs after a period of exceptionally high efficiency and may thus not be related exclusively to the reaction. The mean values of $E_{d.m.}$ at acid and alkaline reaction are not significantly different and similar to the (somewhat too high)

value of 57 mgm. found by Wozak (1929) under field conditions. The values of E_n show that the daily yield of the fixation process often approaches the nitrogen content of the nodules and sometimes exceeds it by as much as one-third. On the average the nodules fix 73 to 80% of their own nitrogen content daily, and at least 89 and mostly 95–96% of this is even in the early stages transferred to the rest of the plant. This rate of transfer is considerably higher than in soy beans and cowpeas, where 20% or more of the fixed nitrogen may be retained by young nodules, as shown by Whiting

TABLE 12.
Nitrogen-fixing Efficiency of Lucerne Root Nodules.

Experiment Number.	Days.	Acid Sand.				Alkaline Sand.			
		pH.	$E_{d.m.}$	E_n.	Trans. %.	pH.	$E_{d.m.}$	E_n.	Trans. %.
1. (Table 3.)	0	5·7	(>53)	(>0·71)	96	6·7	(>44)	(>0·56)	95
	64	5·0–5·7	60	0·85	95	6·9–7·0	68	1·11	96
	92	5·1–5·5	52	0·63	94	7·1–7·2	57	0·67	94
	120	4·6–4·8				7·0–7·1			
2. (Table 4.)	0	5·1	(nil)	(nil)		7·2	(>23)	(>0·30)	89
	60	4·8–5·0	88	1·18	94	7·1–7·2	77	0·94	96
	85	4·6–4·8	30	0·42	95	7·0–7·4	86	1·09	(100)
	100	4·5–4·6				7·2–7·5			
3. (Table 5.)	0	5·4–5·5	(>39)	(>0·58)	94	6·8–7·0	(>47)	(>0·70)	95
	56	6·0–6·2	103	1·36	96	7·3–7·4	62	0·72	94
	76	5·1–5·7	20	0·28	89	7·6–7·8	60	0·83	97
	91	5·1–5·4	11	0·15	(103)	7·2–7·3	41	0·59	98
	105	4·9–5·4				7·2–7·3			
4. (Table 6.)	35	6·1–6·2	43	0·65	91	7·0	42	0·70	92
	68	5·8–6·2	49	0·73	97	7·2–7·5	55	0·80	97
	88	5·4–5·6	44	0·67	93	7·2–7·5	38	0·55	94
	102	5·3–5·6				7·3–7·4			
5. (Table 7.)	0	5·4–5·7	(>34)	(>0·55)	94	7·1–7·3	(>57)	(>0·82)	96
	59	5·4–5·9	90	1·30	97	7·3–7·7	80	1·18	98
	74	5·3–5·6	43	0·59	95	7·0–7·3	46	0·60	96
	88	5·1–5·6	60	0·80	97	7·3–7·5	50	0·67	99
	102	6·0–6·1				7·4–7·6			
Mean ..			53	0·73	95		59	0·80	96

$E_{d\,m.}$ = mgm. nitrogen fixed per day per gm. dry nodule-matter.
E_n = same per mgm. nodule-nitrogen.

(1915), Bond (1936), and Wilson and Umbreit (1937). In one or two instances the nitrogen content of the nodules remains stationary or decreases during the last period, with the result that the transfer equals or exceeds the fixation.

The corresponding figures for subterranean clover are seen in Table 13. Apart from the somewhat abnormal case represented by the final stage of Experiment No. 4, when growth had almost ceased, the average rate of nitrogen fixation by clover nodule tissue appears to be somewhat less than one-half of that of lucerne. The values of $E_{d.m.}$ range from 14 to 41 at acid and 22 to 53 at alkaline reaction, and the means are not significantly different. The E_n-values show, if we again disregard the last stage of Experiment No. 4, that the nodules fix from about one-sixth (Experiment 1, 62–90 days, pH 4·8–5·0) to two-thirds (Experiment No. 3, 96–116 days, pH 7·3–7·5), and on the average one-third, of their own nitrogen content per day. There is again no significant difference between the two ranges of reaction. Owing to the higher proportional weight

TABLE 13.

Nitrogen-fixing Efficiency of Subterranean Clover Root Nodules.

Experiment Number.	Days.	Acid Sand.				Alkaline Sand.			
		pH.	$E_{d.m.}$	E_n.	Trans. %.	pH.	$E_{d.m.}$	E_n.	Transf. %.
1. (Table 8.)	0	5·7				6·7			
			(>17)	(>0·22)	85		(>0·17)	(>0·21)	85
	62	4·8–4·9				6·9–7·1			
			14	0·18	86		20	0·25	85
	90	4·9–5·0				7·0–7·2			
			22	0·24	86		32	0·37	93
	125	4·8–5·0				7·1–7·2			
2. (Table 9.)	0	5·3–5·4				7·1–7·3			
			(>12)	(>0·19)	86		(>9)	(>0·18)	85
	73	5·1–5·6				7·2–7·4			
			33	0·50	92		22	0·35	88
	94	4·7–4·8				7·4–7·5			
			16	0·27	(105)		27	0·43	(103)
	115	4·2–4·8				7·3–7·6			
3. (Table 10.)	0	4·9–5·0				6·8–6·9			
			(>29)	(>0·33)	92		(>19)	(>0·23)	88
	75	6·1–6·2				7·2–7·4			
			30	0·37	84		22	0·25	93
	96	5·9–6·0				7·3–7·5			
			29	0·36	86		53	0·65	93
	116	5·6–5·7				7·3–7·4			
			41	0·55	(104)		23	0·34	92
	130	4·9–5·0				7·2–7·3			
4. (Table 11.)	0	5·8				7·0			
			(>11)	(>0·16)	80		(>13)	(>0·17)	81
	63	5·1–5·2				7·1–7·4			
			20	0·28	92		23	0·32	94
	82	5·0–5·2				7·3–7·5			
			34	0·50	90		29	0·44	90
	96	4·3–4·7				7·1–7·4			
			4	0·07	(105)		6	0·10	89
	110	4·2–4·4				7·5–7·6			
Mean ..			23	0·33	91		26	0·35	90

$E_{d.m.}$ = mgm. nitrogen fixed per day per gm. dry nodule-matter.
E_n = same per mgm. nodule-nitrogen.

and nitrogen content of the clover nodules, the rate of nitrogen transfer is somewhat lower than in lucerne—mostly 85 to 94%, but sometimes exceeding 100% in the later stages when the nitrogen content of the nodules begins to decrease. As a whole the figures for nodule efficiency and nitrogen transfer in subterranean clover are comparable to those found in young soy bean plants by Bond (1936) and Wilson (1940). A somewhat higher efficiency value (55 mgm.) was calculated by Wozak (1929) in red clover under field conditions, but this figure may be regarded as somewhat too high, and also the figures in Table 1 suggest a higher efficiency of nodules in white clover than in subterranean clover. On the other hand the efficiency of red clover nodules in the experiments of Chen and Thornton (1940) appears remarkably low, as mentioned in the introduction, and it might be questioned whether the equal efficiency of "effective" and "ineffective" nodules in equal time and tissue volume, which Chen and Thornton observed, would also exist under conditions permitting a generally higher level of efficiency (cf. Boyes and Bond, 1942, on soy beans).

Effect of Molybdenum on the Efficiency of Root Nodule Tissue.

A supplementary experiment was performed in order to test the possible effect of molybdenum and the influence of reaction on the availability of the reserves of this element in the sand. The following nutrients were given: KH_2PO_4, 0·4 gm., $CaCl_2$, 0·4 gm., $MgSO_4$, 0·2 gm., NaCl, 0·2 gm., $FeCl_3$, 0·04 gm., minor salts (with and without Na_2MoO_4), 1·2 mgm., and 0·1% $CaCO_3$ in the alkaline sand. Each treatment included four replicate pots. Lucerne, six plants per pot, was sown on 31st May, 1946, and harvested after 101 days, and subterranean clover (seven plants) on 30th May, 1946, and harvested after 110 days. The results are shown in Table 14.

The net gains of nitrogen by lucerne are not significantly different, but the gains per gm. dry nodule show a clear effect of both reaction and molybdenum. The highest weight of the nodule-fraction, both absolutely and proportionally, and the lowest gain of nitrogen per gm. dry nodule, are found in acid sand without molybdenum. Addition of molybdenum results in an increase in gain per gm. nodule, significant at the 5% point; a further increase is produced by calcium carbonate, but molybdenum in addition to this is without effect. Determinations of molybdenum by Marmoy's thiocyanate method (Piper, 1942) show approximately 25 p.p.m. Mo in nodule substance from the treatment "+ $CaCO_3$ – Mo", which has a higher nitrogen-fixing efficiency than nodule substance from the treatment "– $CaCO_3$ + Mo" with its 50% higher molybdenum content. It thus appears that in this sand the acid reaction has reduced the efficiency of the nodules *partly* by limiting the uptake of molybdenum, but also in other ways, and that the molybdenum concentration of lucerne nodule tissue necessary for maximum efficiency lies somewhere between 10 and 25 parts per million.

(It should be noted that these figures are probably a little too high. The analyses were made before it was realized that the presence of iron tends to exaggerate the molybdenum figures, as shown by Dick and Bingley (1946). Sufficient material was not available for renewed molybdenum determinations, but analyses of some other materials suggested that the figures should be reduced by some 10 to 20%.)

The net gains of nitrogen by clover are likewise unaffected by either reaction or molybdenum supply, and the gain per gm. dry nodule is lowest in acid sand without molybdenum; this is increased very significantly (beyond the 1% point) by addition of either molybdenum or calcium carbonate, but the reaction *per se* appears to have no effect, inasmuch as the gain per gm. nodule in acid sand with molybdenum is as high as at alkaline reaction. The molybdenum determinations, in which precautions were taken against the effect of iron, show that a content of about 4 p.p.m. Mo is insufficient for maximum efficiency of the nodules, but no further effect results from raising the content above 7–8 p.p.m. Mo. The effect of calcium carbonate on subterranean clover in the absence of added molybdenum thus seems to consist chiefly in rendering the molybdenum of the sand medium available to the plants (cf. Anderson and Oertel, 1946), while in lucerne it also has other effects. The question naturally arises whether this

lower molybdenum requirement of the clover nodule tissue has a causal connection with its generally lower nitrogen-fixing efficiency and its lesser sensitivity to acid reaction in comparison with lucerne.

TABLE 14.

Influence of Hydrogen Ion Concentration and Molybdenum on Nitrogen Fixation by Lucerne and Subterranean Clover.

		Lucerne (31/5/46–9/9/46).				Clover (30/5/46–17/9/46).			
		$-CaCO_3$.		$+CaCO_3$.		$-CaCO_3$.		$+CaCO_3$.	
		−Mo.	+Mo.	−Mo.	+Mo.	−Mo.	+Mo.	−Mo.	+Mo.
pH of sand, initial		5·7	5·7	7·5	7·5	5·7	5·7	7·5	7·5
„ „ „ final		4·7–4·9	4·6–4·9	6·8–7·0	6·6–6·9	4·7–4·9	4·8–5·0	7·0–7·4	7·4–7·5
Dry matter, gm. per pot, mean.	Tops	4·14	4·24	4·71	4·61	6·61	6·83	7·45	7·72
	Roots	3·21	3·49	3·17	3·23	1·99	1·85	1·93	2·02
	Nodules	0·219	0·180	0·124	0·121	0·367	0·287	0·302	0·288
Percentage of N in dry matter.	Tops	3·51	3·76	3·56	3·53	3·07	3·09	2·96	3·05
	Roots	2·19	2·56	2·41	2·33	2·52	2·66	2·33	2·15
	Nodules	6·45	7·20	7·96	7·90	6·98	6·95	6·81	6·84
Molybdenum in dry matter, p.p.m.	Tops	0·4	7·7	1·1	28·1	0·6	13·4	0·9	33·2
	Roots	1·0	8·1	1·4	23·9	1·1	6·7	1·0	12·8
	Nodules	9·6	37·5	24·8	73·7	4·4	7·1	7·4	15·9
Total N in plants, mgm.		229·9	261·8	253·7	247·4	278·4	280·0	286·0	298·8
Net gain of N, mgm.		222·8	254·7	244·5	238·2	268·1	269·7	273·6	286·4
Do. per gm. dry nodule-substance		1040	1496	1974	1974	735	946	906	991
Total Mo in plants, γ		7·0		12·3		7·8		10·9	
Mgm. N per γ Mo		32·8		20·6		35·7		26·2	
Nodules per plant.	Mean	32·4	22·0	46·8	47·1	424	292	314	320
	S.E.	±3·98	±1·91	±2·76	±4·18	±34·0	±18·8	±23·7	±21·9
		Significant difference at P.				Significant difference at P.			
		0·05	0·02	0·01		0·05	0·02	0·01	
Net gain of N		39·6	48·7	55·5		39·4	48·5	55·2	
Do. per gm. dry nodule-substance		401	493	562		108	133	151	

The counts of nodules in both plants show that in acid sand the numbers are significantly lowered by the addition of molybdenum, while the pH-values show no corresponding difference. In the presence of calcium carbonate, which has rendered the molybdenum in the sand more available, the further addition of molybdenum has no such effect on the numbers of nodules. The phenomenon of reduction in numbers of nodules by supply of molybdenum to soil deficient in this element has already been observed by Anderson and Thomas (1946), who also conclude that the effect of molybdenum on legumes consists in stimulation of the nitrogen-fixing activity of the nodule tissue. This contention, although based only on numbers and not on mass of nodules, is fully confirmed by the present results which emphasize that a sufficient supply of available molybdenum is an important factor to be considered in experiments on the effect of reaction on symbiotic nitrogen fixation.

Several earlier experiments on this problem seem to acquire a new aspect in the light of these findings, as well as those of Oertel *et al.* (1946), which strikingly illustrate the effect of molybdenum in widening the pH-limits for growth of subterranean clover. The reduction in the efficiency of subterranean clover nodules in acid sand and soil

observed previously (Jensen, 1943, 1944) might well be due simply to inhibited uptake of molybdenum. The results with lucerne seem also to need reinterpretation. It was observed (Jensen, 1946) that not only the efficiency of the nodules (gain of nitrogen per gm. dry matter), but also the actual yields of nitrogen, are lowered at a content of only 2·7 p.p.m. Mo in the nodule substance, a result which agrees with the fact that the efficiency, but not necessarily the actual yield, begins to suffer at a limit close to or somewhat above 10 p.p.m. Mo in lucerne nodule substance. This may explain why earlier experiments (Jensen, 1943, 1944; cf. also Olsen, 1925) showed that lucerne grown in soil of pH about 5 fixed only about half as much nitrogen as at pH 7·0–7·3; the sand-soil-mixture used for the main experiments with lucerne (1943, Table 8; 1944, Table 2) was the same as used in another experiment (Jensen and Betty, 1943), where nodules of lucerne were found to contain only 3·2 p.p.m. Mo at pH 4·9–5·4 against 10 p.p.m. at pH 7·5–8·0. Such a difference in the molybdenum content would be quite likely to have a considerable effect on the gain of nitrogen, apart from the difference in reaction.

The records of several other investigations on the growth of legumes in soil, sand, or water culture at different reaction, discussed by Fred *et al.* (1932) and Wilson (1940), seem also to admit the possibility of molybdenum deficiency at the lower pH-ranges. In sand culture experiments with soy beans, Hopkins (1935) found that pH-values below 6 were unfavourable for growth with free nitrogen, although not with nitrate, and gave rise to pathological symptoms (chlorotic spots on the leaves) which were suggestive of molybdenum deficiency and disappeared when the reaction was corrected. Alway and Nesom (1927) observed in field trials with lucerne on acid soil (pH 5·0–5·7) a far greater benefit from soil transfer than from pure culture inocula which were effective only when lime was applied. The authors suggest as one possible explanation the presence in the inoculating soil of small amounts of some chemical compound beneficial to the bacteria. The possibility of this active element being molybdenum suggests itself.

General Conclusions.

If we try to form a general picture from the preceding experiments, we find first that the reaction of the medium, as in previous experiments (Jensen, 1943), shows different effects on the *formation* and on the *development* and *activity* of the root nodules. In lucerne the numbers of nodules decrease at acid reaction, particularly in the pH-interval from about 6 to 5, but in subterranean clover the numbers are highest at pH 4·5–5·5 and tend to decrease at neutral or faintly alkaline reaction. This may be regarded as showing the effect of reaction on the ability of the nodule bacteria, existing outside the plants, to invade the roots and form nodules. *Rhizobium trifolii* thus appears far more acid-tolerant than *Rhizobium meliloti*, and this agrees to some extent with the behaviour of these bacteria *in vitro* (Jensen, 1942). However, the acid-tolerance in sand medium appears considerably greater; lucerne forms nodules at pH 5 and less (cf. Olsen, 1925, who found sparse nodule formation in soil of pH 4·0–4·4), and a pH-range of 4·5–5·0 is unfavourable for survival of *Rhizobium trifolii* in soil (Bryan, 1923; Wilson, 1926, 1931) and prohibitive to growth in pure culture (Snieszko, 1928; Jensen, 1942), although not to nodule formation in agar medium (Jensen, 1942). Further investigations on this point are needed.

The mass of root nodule tissue is little affected by the reaction because, especially in lucerne, the smaller number of nodules in acid sand is compensated by increased size of individual nodules. The same applies to the nodular efficiency, expressed as gains of nitrogen per gm. dry matter, which in lucerne shows definite decreases only at pH 5 and less, and which in subterranean clover remains unaffected at pH 4·5–4·8 (adequate molybdenum supply presupposed). This comparative insensivity of the nitrogen fixation process to the reaction of the external medium must evidently be ascribed to the fact that once the nodule bacteria have gained entry into the plant, the external reaction can only influence their activities through its effect on the assimilation of mineral nutrients and on the internal reaction of the root and especially the nodule tissue. However, the intranodular reaction appears within wide limits

independent of that of the growth substrate (Jensen, 1943), and the present experiments with combined nitrogen, especially Tables 7 and 11, show no evidence of inhibited uptake of minerals, as growth was equally good at acid and alkaline reaction.

The lack of correlation between the numbers of root nodules and the resulting nitrogen fixation must be viewed on the background of the fact that a heavy inoculum of nodule bacteria was provided in all cases. A certain minimum number of nodules may be regarded as necessary for maximum fixation, since there would obviously be limits to the ability of the plant to compensate for the lower number of nodules by increased nodule size, and in a medium harbouring a sparse population of *Rhizobium* an unfavourable reaction might well prevent this number from being reached. A complete answer to the question of interrelation between the reaction of the medium, the number and the mass of nodules, and the efficiency of the nodule tissue could probably be obtained by growing the plants at several constant pH-levels (flowing nutrient solution) and periodically determining the intranodular pH as well as increases in dry matter and nitrogen. By altering the reaction of the medium during growth it should be possible to define the pH-limits at which not only the formation of new nodules but also the activity of those already formed is completely inhibited.

The provision of combined nitrogen in quantities similar to, or somewhat higher than, the amounts fixed in the free-nitrogen series has comparatively little influence on the actual numbers of nodules, but tends to lower the numbers in proportion to the weight of the roots. The average size of the nodules is greatly diminished, and the proportional weight of the nodule fraction is reduced to roughly one-fourth of its value in the free-nitrogen series, as shown by the following summary of the weight of nodules in percentage of total dry matter:

	Lucerne. (Tables 5–7.) %	Subterranean Clover. (Tables 10–11.) %
Free N	1·08–4·23	2·35–6·65
Combined N	0·26–1·16	0·27–1·73

At the same time the nitrogen fixation almost ceases although it may continue if the combined nitrogen is used up. The inhibitory effect of combined nitrogen on nitrogen fixation seems due to reduced development of nodule tissue, as found by Nicol and Thornton (1936), as well as to lower efficiency of the tissue formed, probably because the available carbohydrates are largely used for protein synthesis with the combined mineral nitrogen (cf. Wilson, 1940). There is no evidence that the total amount of root and nodule substance in proportion to the tops is decreased by combined nitrogen, as stated by Allison and Ludwig (1934).

The data on the nodule efficiency in Tables 12 and 13 may permit some tentative considerations on certain aspects of the mechanism of nitrogen fixation and transfer. Bond (1936) concluded from experiments with soy beans that the fixation is a kind of respiration process which results in a steady transfer of some 80–90% of the fixed nitrogen from the nodules to the rest of the plant. Wilson and Umbreit (1937), on the other hand, thought that the observations might equally well be explained on the basis of the views commonly held before, that transfer takes place through disintegration of the bacteria in the nodules by proteolytic enzymes of the host cells, autolysis, or a bacteriophage, and subsequent transport of the soluble nitrogenous digestion products.

The present experiments, particularly with lucerne, show a turnover of nodule-nitrogen considerably more rapid than in soy beans. If we assume with Bond (1941) that the bacterial tissue accounts for roughly one-half of the whole nodule, the values of $E_{d.m.}$ (Tables 12 and 13), which should properly refer to dry bacterial tissue, would be approximately doubled. The bulk of the nodule-nitrogen is in the bacterial tissue,

and the perifereal tissue may probably without grave error be assumed to have the same gross chemical composition as the rest of the root substance (cf. Bond, 1941). The analyses in the preceding tables show that in lucerne grown with free nitrogen the ratio (%N in nodules)/(%N in roots) varies between 2·77 and 6·14, with a mean value of 3·35. The corresponding figures for clover, apart from two abnormal cases in Table 8, 62 days, are 2·33, 5·99, and 3·15. This indicates that on the average the perifereal tissue contains 23–24 and the bacterial tissue 76–77%, or roughly three-fourths, of the total nodule nitrogen. A calculation on this basis would raise the values of E_n in Tables 12 and 13 by one-third; the nitrogen content of the bacterial tissue in lucerne would then on the average renew itself in about 24 hours, and in clover in somewhat more than two days. However, the bacterial tissue consists of *Rhizobium*-cells ("bacteroids") and host cells, of which the relative weights are not known, but the former have probably the higher nitrogen content. We may perhaps assume that the bacteria contain two-thirds of the bacterial-tissue nitrogen or one-half of the total nodule nitrogen; the E_n values calculated on the basis of bacteria alone would then be doubled. Provided that the bacteria and not the host cytoplasm are the site of nitrogen fixation, and that the transferred nitrogen is derived from dead bacterial cells only, the average E_n values in lucerne (Table 12) would imply a renewal of the whole bacterial nodule population every 15 or 16 hours, in extreme cases (Experiment No. 3, acid sand, 56–76 days) even every 9 hours. While such a rate of reproduction may be conceivable, it appears unlikely in view of the generally low reproductive capacity of bacteroids taken from nodules (Fred *et al.*, 1932; Almon, 1933). The rate of nitrogen turnover would thus seem to fit better with Bond's idea of transfer of a nitrogenous compound secreted by the bacteria during active metabolism. This seems also supported by the fact that when nitrogenous root secretion occurs, it takes the form of an apparent key-compound of the nitrogen fixation process: aspartic acid or its derivative β-alanine (Virtanen, 1938), or, according to more recent evidence (Virtanen *et al.*, quoted by Wilson and Burris, 1947), a mixture of glutamic acid, aspartic acid, and β-alanine.

It also seems entirely possible that the transferred nitrogen may originate from two sources: excess of a key-compound of the nitrogen fixation process (glutamic acid?), and digestion products of bacteroids dying during the life history of the nodule population. The actual existence of the last process seems indicated by the fact that the total nitrogen content of the nodule-fraction often decreases during the later stages of the plant's life. Investigations on the almost untouched problem of the rise and fall of the bacterial population in the nodules might contribute to assessing the relative importance of these two processes. The present evidence would seem to favour the "secretion" rather than the "digestion"-hypothesis.

Summary.

Pot experiments with pasture legumes, mostly lucerne and subterranean clover, were conducted in sand of acid and faintly alkaline reaction. The rate of nitrogen fixation by the root nodule tissue was measured by determining the dry weight and nitrogen content of the separate fractions of tops, roots and root nodules at successive stages of growth.

Root nodules of lucerne fixed from 11 to 103 (average, 56) mgm. nitrogen per gm. dry matter per day, or 0·15 to 1·36 times their own nitrogen content. Significant decreases in nodule-efficiency at acid reaction were only observed in sand of pH near or below 5, but fixation still took place at pH 4·5–4·8. The net gain of nitrogen per pot also as a rule declined significantly only at pH about 5 or less. Generally 94 to 98% of the fixed nitrogen was transferred from the nodules to the rest of the plant.

Nodules of subterranean clover showed a consistently lower rate of activity. Under conditions of vigorous growth 14 to 53 mgm. nitrogen were fixed per day per gm. dry matter, or 0·18 to 0·65 times the nodule nitrogen content. Mostly 80 to 94% of the fixed nitrogen was transferred to the rest of the plant. The fixation per gm. dry nodule was not significantly influenced by the reaction. The net gain per pot sometimes showed decrease at pH about 5 and less, but nitrogen was still fixed in sand of pH 4·2–4·5.

The numbers of root nodules in lucerne decreased conspicuously in sand of pH about 5·5 and less. This decrease was associated with an increase in the size of individual nodules. Nodules of subterranean clover were, on the other hand, usually more numerous in acid than in alkaline sand, and the influence of the reaction on their size was less conspicuous than in lucerne. No correlation was found between the numbers of nodules and the resulting nitrogen fixation, which is determined by the aggregate mass rather than the numbers of nodules.

Combined nitrogen supplied as nitrate or ammonia in quantities comparable to the amounts of nitrogen fixed in the same time had relatively little effect on the numbers of nodules, but reduced their weight and nitrogen-fixing efficiency greatly. Both lucerne and subterranean clover grew nearly equally well at pH 4·5–5·0 and at pH 7·0–7·5 when given combined nitrogen.

A molybdenum content of lucerne nodules between approximately 10 and 25 p.p.m. dry substance appeared necessary for maximum nitrogen-fixing efficiency of the nodule-tissue. The results of earlier experiments suggest that the actual gain of nitrogen decreases when the nodule substance contains only 3 to 10 p.p.m. molybdenum. In nodules of subterranean clover a molybdenum content between 4 and 8 p.p.m. was required for full efficiency per gm. nodule, but the actual gain of nitrogen did not decrease when the nodules contained only 4 p.p.m. molybdenum.

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